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ORIGINAL PAPER

Over-expression of an arabidopsis family A sucrose phosphate synthase (SPS) gene alters plant growth and fibre development

Ji-Young Park · Thomas Canam · Kyu-Young Kang · David D. Ellis · Shawn D. Mansfield

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Abstract The objective of this study was to manipulate the intracellular pools of sucrose by differentially expressing exogenous sucrose phosphate synthase (SPS) and investigating its role in regulating plant growth and fibre development. Tobacco (*Nicotiana tabacum* cv. Xanthi) plants were transformed with an arabidopsis SPS gene under the regulation of the ubiquitously expressed tandem repeat of the 35S cauliflower mosaic virus promoter, and subject to growth trials and fibre characterization. It was apparent that over-expression of SPS resulted in substantially elevated concentrations of sink sucrose pools compared to wild-type plants, while source tissue sucrose pools remained the same. All transformed plants had significantly increased stem height, which was ascribed to internode elongation, and greater stem diameters, longer fibers and increased total dry biomass relative to the control plants. Difference in the chemical composition of either the storage or structural carbohydrates of the wild-type and SPS transgenic lines were only minor. The correlation between increased stem sucrose content and plant phenotypes with elevated SPS gene expression confirm a role for sucrose availability in controlling plant growth and fibre elongation.

Keywords Sucrose phosphate synthase (SPS) · Carbohydrate metabolism · Plant growth · Plant metabolism · Soluble carbohydrates · Sucrose · Tobacco · Fibre development

J.-Y. Park · T. Canam · K.-Y. Kang · S. D. Mansfield (✉)
Department of Wood Science, University of British Columbia, 2424 Main Mall, Vancouver, BC, Canada V6T 1Z4
e-mail: shawn.mansfield@ubc.ca

D. D. Ellis
National Center for Genetic Resources Preservation, USDA-ARS, Fort Collins, CO 80521, USA

Introduction

Sucrose, the major product of photosynthesis, can either be utilized directly by glycolysis or be translocated within the plant as a soluble carbohydrate via the phloem. When imported into sink tissues, sucrose is used for the maintenance of cellular metabolism, cell wall biosynthesis, and respiration or converted to starch for storage and used at a later time (Sturm, 1999; Kutschera and Heiderich, 2002). The reaction catalyzed by sucrose phosphate synthase (SPS, EC 2.4.1.14), the synthesis of sucrose-6-phosphate from fructose-6-phophate and UDP-glucose, is a key regulatory step in the control of sucrose synthesis in plants (Stitt et al., 1988). Intracellular pools of sucrose are subject to the catabolic effects of invertases and/or sucrose synthase (SuSy) to ultimately liberate fructose and glucose (UDP-glucose in the case of SuSy) residues. SPS therefore plays an important role in carbon

partitioning in the regulation of starch production versus sugar accumulation in many physiological and developmental processes such as water stress (Geigenberger et al., 1997), diurnal carbohydrate allocation within the plant (Huber and Huber, 1996; Chen et al., 2005), as well as during flower (Baxter et al., 2003), fruit (Laporte et al., 2001), and cell wall development (Haigler et al., 2001).

A significant amount of the research investigating the role of SPS in photosynthetic tissues has been conducted, and therefore many aspects of its function and regulation in these tissues are known (Foyer et al., 2004; Huber and Huber, 1996; Lunn and MacRae, 2003). In contrast, the role of SPS in nonphotosynthetic tissues, where sucrose import and degradation occurs, is just now becoming clear. In nonphotosynthetic tissues, it has been proposed that SPS may serve two possible roles: the resynthesis of sucrose after import via apoplastic cleavage or direct involvement in carbohydrate regulatory cycles in which sucrose and/or starch are degraded and sucrose is resynthesized via SPS from one or more products (Geigenberger et al., 1997). Examples of nonphotosynthetic tissues expressing SPS include developing endosperm in maize (Im, 2004), potato tubers (Geigenberger et al., 1999), sugar cane stems (Zhu et al., 1997), and kiwi roots (Fung et al., 2003).

Cellulose, the major structural polymer in the plant stems, is an irreversible carbon sink. As such, how plants control carbon partitioning to cellulose biosynthesis is a key question for researchers globally (Haigler et al., 2001). Recent models propose that the substrate for cellulose synthesis, UDP-glucose, is channeled to the cellulose synthase complex via the enzyme SuSy (EC 2.4.1.13) which catalyzes the cleavage of sucrose to generate UDP-glucose and fructose (Delmer et al., 1999; Haigler et al., 2001). In this proposed model, the removal of fructose from the cell is critical for continued and maintained cellulose synthesis, as fructose is a known strong inhibitor of SuSy activity (Haigler et al., 2001). The cycling of fructose to generate sucrose by SPS (fructose-6-P + UDP-glucose) therefore provides a continuous substrate for SuSy and consequently cellulose biosynthesis in the form of UDP-glucose, while concurrently limiting the intracellular pool of fructose (Delmer, 1999). In the context of secondary cell wall formation, SPS is integral, as it has a dual function—contributing to the pathway leading to the synthesis of the precursor substrate (UDP-glucose) for cellulose deposition, as well as maintaining a constant supply of substrate by recycling the products from the initial photosynthate.

The role of SuSy in cellulose synthesis has been well supported by reports of increased transcription and SuSy activity with the onset of secondary cell wall synthesis (Salnikov et al., 2001), as well as immunolocalization studies showing the colocalization of SuSy with tracheary secondary cell wall thickenings (Haigler et al., 2001). Correlated with the increase in SuSy activity during secondary cell wall biosynthesis is growing evidence for concurrent increases in SPS activity. Using three well-established model systems for secondary cell wall synthesis, zinnia (*Zinnia elegans)* tracheary elements, kidney bean (*Phaseolis vulgaris*) etiolated hypocotyls, and cotton (*Gossypium hirsutum*) fibres, Babb and Haigler (2001) showed a correlation between SPS activity and secondary cell wall formation. Furthermore, when glucose is supplied as the sole carbon source to developing cotton fibres, sucrose is synthesized in the fibres, and both SPS and cellulose synthesis activity are increased. Further evidence has been provided by the observation that the increase in SPS activity in cotton fibres is associated with the onset of secondary cell wall formation (Haigler et al., 2001)

In the present study, we investigated the effects of over-expressing an arabidopsis family A SPS gene in tobacco to help clarify the role(s) of SPS in plant growth and carbohydrate allocation during active secondary wall development. While many of the previous studies investigating the differential expression of a heterologous SPS gene have focused on the activity of the maize SPS transgene in photosynthetic tissues using a ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit promoter (SSU), we chose to over-express an arabidopsis SPS gene and focus our efforts on stem-tissue modifications in attempts to better understand the relationship between carbon allocation and cellulose accumulation during secondary wall development in plants.

Materials and methods

Cloning of SPS cDNA

The arabidopsis family A SPS (AtSPS) gene (*At5g20280*) was targeted from the four known SPS

genes in arabidopsis because of its high levels of expression in all tissues (Langenkämper et al., 2002), and cloned using polymerase chain reaction (PCR). Total RNA was isolated from arabidopsis seedlings using Trizol (Gibco BRL) and the cDNA was synthesized with dT_{16} oligonucleotides employing the superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). The 3303 bp DNA fragment was amplified with arabidopsis SPS gene-specific forward (5′-CGATTCTCGATCTTTGATCGTCCCACC-3′) and reverse primer (5′-CCGGCAGCTTATGTACGA CGAAGTA-3′), based on the arabidopsis genome sequence (www.arabidopsis.org). This PCR fragment was cloned into a pCR-Blunt II -TOPO vector (Invitrogen), and the nucleotide sequence confirmed to be identical to the *At5g20280* genebank sequence accession.

Construction of the SPS Plant Expression Vectors

The 2 × 35S promoter fused with an alfalfa mosaic virus untranslated leader sequence (Datla et al., 1993) and the NOS terminator were excised from pBI-426 and ligated into the *Hind*III–*Bam*HI and *Sac*I–*Eco*RI regions respectively, of the pUCAP cloning vector (van Engelen et al., 1995). The 2 × $35S^{prom}$:nos^{term} cassette was excised as a *Hind*III–*Eco*RI fragment and ligated into the pCambia1390 binary vector (Cambia^{TM}). The 2 × $35S^{prom}$:nos^{term} intermediary construct is referred to as pSM1.

The *At5g20280* SPS coding region including the *Bgl*II and *Sma*I restriction sites at the start and stop codon, respectively, was produced by PCR amplification using Pfu DNA polymerase with 5′-AGATCT ACAAGATGGCCGGGAACGAT-3′ and 5′-AGGTT CCCGGGTCAGTCCTTGAG-3′ as forward and reverse primers. The resulting 3185 bp DNA fragment was subcloned using the Zeroblunt TOPO PCR cloning kit (Invitrogen). After excising the *Bgl*II and *Sma*I fragment from the cloning vector, the fragment was ligated into the *Bam*HI and *Sma*I site of the pSM1 binary vectors. Proper ligation and alignment was confirmed by sequence analysis of the DNA junctions. The resulting construct pSM1SPS, was transformed into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) and introduced into *Nicotiana tabacum* cv. Xanthi by leaf disc transformation following a standard procedure (Horsch, 1985).

Briefly, the agrobacterium was incubated overnight in liquid Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 3% sucrose (MS + 3%) and 100 μM acetosyringone. Leaf disks were cut and cocultured with the agrobacterium, shaken for 1 h at room temperature at 100 rpm, blotted dry and plated abaxailly onto MS + 3% supplemented with 0.1 μM each α-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BA), and solidified with 3% (w/v) agar and 1.1% (w/v) phytagel (MS + NAA/BA). After 3 days the discs were transferred to MS + NAA/BA supplemented with carbenicillin disodium (500 mg/L) and cefotaxime sodium salt (250 mg/L). Following three additional days of selective growth, the discs were transferred to MS + NAA/BA containing carbenicillin, cefotaxime, and hygromycin (20 mg/L). After two consecutive 5-week transfers on this media, single shoot tips were transferred to MS + 3% agar containing no antibiotics.

Plants were confirmed as transgenic by PCR screening of genomic DNA using gene-specific oligonucleotides (forward 5′-GGCTATCGTTCAAG ATGCCTCTG-3′ and reverse 5′-AGGCCTCGCAAG GGCAAGTA-3′). Genomic DNA was isolated using the Red Extract and Amp Kit (Sigma, St Louis Mo).

All shoot cultures, including transgenic and nontransformed control lines, were maintained on solid MS + 3% in GA-7 vessels at 22°C under a 16-h photoperiod with an average photon flux density of 40 μmol m^{-2} s^{-1}. Plants were maintained by transferring apical regions at 4-week intervals.

Plant Growth Analysis

Plantlets grown in tissue culture were transferred to 7.5-L pots containing a 50% peat, 25% fine bark, 25% pumice soil mixture in the glasshouse, and covered with 16 oz clear plastic vessels for 1 week to aid in acclimation. Each line, transgenic (4) and control was represented by 10–16 individual plants. Plants were placed under an 18-h photoperiod, with supplemental overhead lighting when ambient intensity was less than 300 μmol m^{-2} s^{-1}. The glasshouse plants were harvested at the onset of flowering as indicated by the formation of flower buds. Plant height (from flower apex to base) and stem diameter were measured at the onset of flower bud formation prior to harvest. Developmental stages of tissues were

standardized by employing a plastichron index, where leaf plastichron index PI = 0 was defined as the first leaf greater than 7 cm in length from the apex, and where PI = 1 is the leaf immediately below PI = 0. A portion of the stem from each plant, spanning PI = 5 to PI = 15, was excised and immediately weighed for total stem fresh weight and biomass measurements. This same section (PI = 5 to PI = 15) was dried at 105°C for 48 h for dry weight determination, and retained for further analysis. The lower section of the stem (below PI = 15) was dried at room temperature for fibre quality analysis. All data was analyzed using one-way ANOVA ($\alpha = 0.05$) and Scheffé post hoc tests.

Quantitative Real-Time PCR

Total RNA was isolated in triplicate from PI = 3 and PI = 4 leaves, as well as the internode (stem) between these leaves. Tissue was ground in liquid nitrogen using a mortar and pestle, and total RNA was extracted using Trizol reagent according to the manufacturer's instructions. An equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1), pH 6.7, were added to DNase-treated RNA. The samples were then vortexed and centrifuged for 5 min at 13,000 rpm. Ten microlitres aliquots of 3 M sodium acetate (pH 4.5) and 200 μL of 100% ethanol were added to the supernatant, incubated at -80°C for 1 h and centrifuged at 13,000 rpm for 15 min at 4°C. The RNA pellet was reprecipitated in 500 μL 75% ethanol, centrifuged at 13,000 rpm for 10 minutes at 4°C, and resuspended in 50 μL RNase-free distilled water after air drying.

One microgram of total RNA was used for cDNA synthesis using dT_{16} oligonucleotides with Superscript II Reverse Transcriptase (Invitrogen) following manufacturer's instructions. AtSPS transcript abundance was quantified with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) on an Mx3000P Real-Time PCR System (Stratagene), employing AtSPS-F3 (5′-CCACAGTGGCAAAGTG ATGATGGC-3′) and AtSPS-R4 (5′-TCTGACCTCT CCAGTGATCCC-3′) as forward and reverse primers, respectively. Thermocycler conditions for all real-time analyses were: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s. Transcript number was calculated using the Mx3000P v1.20 software (Stratagene) with regression equations generated from a serial dilution of precisely known concentrations of plasmid DNA containing the arabidopsis SPS gene.

SPS Enzyme Activity

Discs (1.1 cm^2) from four fully expanded leaves isolated between PI = 3 to PI = 5, and 1.5 cm stem segments harvested between internode PI = 2 and PI = 3 from each of three different plants per transgenic line and control plants were employed for SPS enzyme activity determination. Frozen samples were ground in liquid nitrogen with 1.8 mL of extraction buffer containing 50 mM HEPES–KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, 2 mM aminocaproic acid, 0.5 mM PMSF, 0.1% TritonX-100, and 10% glycerol. Samples were then transferred to prechilled Eppendorf tubes and centrifuged at 4°C, 12,000*g* for 15 min. About 1.5 mL of the supernatant was then desalted on a Econo-Pac® 10DG column (BioRad, Hercules CA), from which 100 μL was immediately used for the SPS assay.

SPS activity (V_{max}) was assayed as described by Iraqi and Tremblay (2001) and Baxter et al. (2003). In short, samples were incubated for 20 min at 25°C in 50 μL of reaction buffer (50 mM HEPES–KOH pH 7.5, 20 mM KCl, and 4 mM $MgCl_2$) containing 12 mM UDP-glucose, 10 mM fructose-6-phosphate, and 40 mM glucose-6-phosphate. The reaction was terminated by incubation at 95°C for 10 min with 70 μL of 30% KOH to destroy any unreacted hexose phosphates and the samples were centrifuged at 4°C, 12,000*g* for 5 min. After adding four volumes of 0.14 (w/v) anthrone reagents (in 14.6 M H_2SO_4) the reaction absorbance was measured at 620 nm. A standard curve spanning 0–200 nmol sucrose prepared in the assay medium was used to calculate absolute amounts of sucrose-6-phosphate generated. Controls containing boiled protein extract were included to provide appropriate blanks. Total protein content was measured according to Bradford (1976).

Cell Wall Chemical Analysis

The chemical composition of stems was determined according to a modified micro-klason analysis (Huntley et al., 2003). In brief, ~0.5 g of freeze-dried stems were ground to pass a 40-mesh screen using a Wiley mill, soxhlet-extracted with acetone for 6 h, digested

with 72% H_2SO_4 for 2 h, and then hydrolyzed in 4% H_2SO_4 for 1 h at 121°C. The total weight of nonhydrolyzed, extracted components was determined gravimetrically (acid-insoluble lignin), while the filtrate was analyzed for acid-soluble lignin by absorbance at 205 nm according to TAPPI Useful Method UM250. The carbohydrate composition of the treatment filtrates, expressed in the anhydro-form, was determined by high performance anion-exchange chromatography on a CarboPac PA-1 column using a Dionex High Pressure Liquid Chromatography (HPLC) system (Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector. Carbohydrates were expressed as μg/mg of the dry weight of the sample.

Soluble Sucrose and Starch Analyses

Stems and leaves were harvested and immediately frozen in liquid nitrogen and freeze-dried for 48 h. Tissue (20–50 mg) was ground with a mortar and pestle in liquid nitrogen, and incubated for 24 h at -20°C with 4 mL of methanol/chloroform/water (12:5:3). The samples were centrifuged for 10 min at 6,000 rpm and 4°C, and the supernatant collected. The pellet was washed with 8 mL (2 × 4 mL) of methanol/chloroform/water (12:5:3), centrifuged for an additional 10 min at 6,000 rpm and 4°C, and the supernatants pooled. An aliquot of 5 mL of distilled water was added to the pooled supernatants and phase partitioned, and 1 mL of the resulting aqueous phase containing the soluble sugars was dried in a speedvac at 40°C. The pellet was re-suspended in 200 μL of nanopure water and filtered through a nylon filter (0.45 μm). The soluble sucrose concentrations were quantified by anion exchange HPLC. Sugars were eluted with water at room temperature at a flow rate of 1 mL/min. Sucrose concentrations were determined using regression equations from calibration curves that were derived from external standards.

The residual pellet was hydrolyzed using 4% sulphuric acid at 121°C for 4 min. Starch content was quantified by the liberation of glucose which was directly quantified by HPLC employing similar conditions as described above.

Fibre Quality Analysis

To determine the fibre length, a portion of the lower stem of tobacco plants (below PI = 15) was cut into representative samples of approximate dimensions of 2 mm × 2 mm × 30 mm, and reacted in Franklin solution (1:1, 30% peroxide:glacial acetic acid) supplemented with 3.6% sodium hypochlorite for 24 h at 70°C. The solution was decanted and the remaining fibrous material was reacted in pure Franklin solution for an additional 48 h at 70°C. The solution was again decanted and the fibrous tissue washed under vacuum with deionized water until a neutral pH was achieved. The samples were then resuspended in 10 mL of deionized water, and diluted appropriately to obtain a count of 25–40 fibres s^{-1} on a Fibre Quality Analyzer (Optest). All samples were run in triplicate.

Results

Plant Regeneration

Transformed tobacco plants regenerated from the AtSPS-agrobacterium treated leaf explants displayed no morphological abnormalities or deleterious growth effects. Following isolation of single shoots representing individual unique transformed lines on media supplemented with hygromycin, AtSPS integration was confirmed by genomic PCR screening to amplify a diagnostic fragment specific to the AtSPS gene. Four independent transgenic lines selected from 14 confirmed 2 × 35S:SPS transformants (based on high real-time qPCR expression levels of tissue culture grown plants) and corresponding control (wild-type) shoots were propogated *in vitro* as shoot cultures, and individual shoots of all lines were rooted and transferred into the glasshouse as described.

SPS Transcription Abundance

AtSPS transcript abundance (transcript copy number/μg total RNA) for both leaf and stem tissue was quantified in all transformed lines (Figure 1). No amplification product was observed in the nontransformed control tissues with the primer pair employed for RT-PCR, and therefore all transcript measured was due to the expression of the exogenous family A AtSPS transgene. As expected, AtSPS transgene expression varied quantitatively by tissue type and between the transgenic lines. The transcript abundance in the leaf tissues was comparable among the

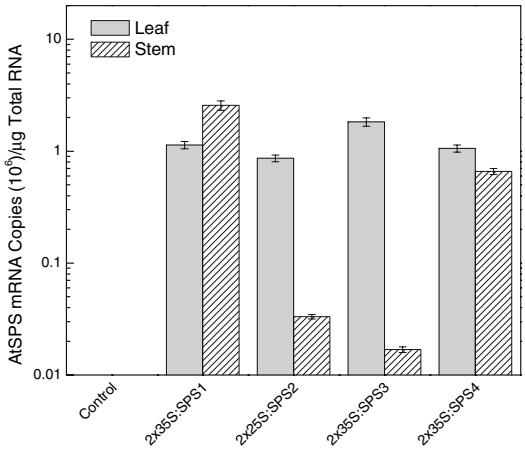

Fig. 1 AtSPS mRNA transcript levels in transgenic and nontransgenic control tobacco plants. One microgram total RNA from PI = 3/PI = 4 leaves and whole stems tissue was analyzed by quantitative RT-PCR. AtSPS mRNA copy numbers were calculated from the standard curve generated with dilution series of SPS cloning vector plasmid DNA. Data are means of three replicates from individual leaves or stems ± SD of each of five plants per independent transgenic line and control plants

transgenic lines, with two lines displaying roughly equal transcript abundance in the stem tissue. In contrast, two lines (2 × 35S:SPS2 and 2 × 35S:SPS3) had substantially lower transcript levels in the stems compared to the other transgenic lines.

SPS Enzyme Activity

Two transgenic lines were randomly selected for SPS enzyme activity determination and showed a clear trend for increased SPS activity (on average as much as 2×) relative to the nontransformed controls (Table 1). Although both lines examined showed an increase in SPS enzyme activity compared to the nontransformed controls in both stem and leaf tissue the greatest increases were observed in the leaf tissue. It was also apparent that the native SPS activity in transgenic and control tobacco plants seems to also be naturally higher (50%) in leaf tissue when compared to stem tissue from the same plant. There appears to be an observable correlation between SPS enzyme activity and all of the evaluated growth parameters, where transgenic line 2 × 35S:SPS1 displays greater stem height, larger diameter, more total biomass and longer fibres than transgenic line

Table 1 Average AtSPS enzyme activity in leaves (PI = 3 to PI = 6) and stem sections (PI = 2) of transgenic and nontransgenic control tobacco plants

	Stem SPS Activity (nmol sucrose/mg protein/min)	Leaf SPS Activity (nmol sucrose/mg protein/min)
Control	88	135
2 × 35S:SPS1	207	321
2 × 35S:SPS4	177	214

Data are means of three replicates per line

2 × 35S:SPS4, and also had the higher SPS enzyme activity in both leaf and stem tissue. Unfortunately, the full complement of lines was not analyzed for SPS activity and therefore we do not have the benefit of a comprehensive comparison. We have, however, generated a T_1 generation of these same constructs, as well as transgenic tobacco simultaneously expressing SPS and sucrose phosphate phosphatase (SPP) together under the regulation of a constitutive viral promoter (2 × 35S:SPS–SPP). These plants are the focus of our continuing efforts to understand the role of sucrose metabolism and carbon allocation on plant growth and fibre formation, and will include a thorough analysis of enzyme activity in relation to plant growth rates, as well as interaction(s) between these two key sucrolytic enzymes via bioluminescence resonance energy transfer (BRET) assays.

Soluble Sucrose Concentration and Starch Content

Although AtSPS transcript abundance and SPS activity was higher in leaf tissue, generally the sucrose content in the leaves was slightly reduced when comparing the SPS-transgenic and the control plants. In contrast, relative to the nontransformed control plants, the level of sucrose in the stem was considerably higher in all four of the 2 × 35S:SPS lines (Table 2). The change in stem sucrose levels in the transgenic lines relative to the controls ranged on average from ~88% (2 × 35S:SPS3) to over a twofold increase (118% in 2 × 35S:SPS1).

An evaluation of storage polysaccharide following extraction of the soluble sugars demonstrated a reduction in stem starch content in all transgenic lines (Table 2), despite the substantial pools of soluble sucrose. The observed reduction in starch

Table 2 Soluble sucrose concentrations and starch content of transgenic and nontransgenic control tobacco plant tissue

	Stem Tissue		Leaf Tissue	
	Sucrose (μg/mg)	Starch (mg/g tissue)	Sucrose (μg/mg)	Starch (mg/g tissue)
Control	7.20 (1.31)	3.06 (0.31)	4.59 (0.33)	7.87 (2.09)
2 × 35S:SPS1	15.74 (1.25)	2.63 (0.18)	2.44 (1.26)	5.61 (0.65)
2 × 35S:SPS2	14.36 (1.57)	2.42 (0.13)	3.46 (0.81)	5.93 (1.37)
2 × 35S:SPS3	13.50 (0.20)	2.16 (0.49)	2.68 (1.51)	6.56 (1.45)
2 × 35S:SPS4	13.57 (2.73)	2.30 (0.12)	4.82 (1.13)	7.20 (0.26)

Data are means ± SD of five independent plants per line

content ranged from decreases of 15–30%. In general, a similar trend was observed in leaf tissue, were starch content was shown to be slightly reduced compared to the control plants. Interestingly, the soluble sucrose content of the leaf tissue was also slightly reduced in the transgenic lines compared to the wild-type plants.

Cell Wall Chemistry Analysis

Cell wall structural constituents indicated that all the transgenic tobacco lines had reduced total cell wall lignin content (Table 3), and this reduction was primarily accounted for by a reduction in the acid insoluble lignin fraction (data not shown). Total lignin content was reduced by as much as ~12% (2 × 35S:SPS3) compared to the control plants. In contrast, all AtSPS tobacco lines had elevated concentrations of acetone extractives. This may be ascribed to higher free phenolics, however, such an analysis warrants further investigation. Similar to the cell wall lignin content, all the structural carbohydrates (cellulose and hemicellulose) were slightly reduced in the AtSPS transgenic lines compared to the wild-type plants (Table 3). The general reduction in structural cell wall chemistry is likely related to the observed enhanced growth characteristics, i.e. enhanced rate of fibre elongation (growth) may possibly result in a reduction in the overall extent of secondary wall deposition, and therefore would result in an overall reduction in cell wall constituents. However, this quantification warrants further investigation.

Plant Growth

The four 2 × 35S:SPS transgenic lines selected for growth trials were shown to have significantly greater stem height at the onset of flowering than the corresponding nontransformed controls (Figure 2). The altered stem height was as much as 50%, and generally comparable among the transgenic lines evaluated. Furthermore, the time to maturity did not vary between the transgenic lines and the

Table 3 Cell wall chemistry (lignin and carbohydrate) concentrations of transgenic and nontransgenic control tobacco stem tissue

	Extractives (mg/100 mg)	Total lignin (mg/100 mg)	Structural carbohydrates (μg/mg)					
			Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose
Control	3.36 (0.48)	22.53 (0.42)	8.76 (0.93)	5.66 (0.55)	10.04 (0.55)	325.15 (10.68)	126.35 (6.21)	19.10 (0.67)
2 × 35S:SPS1	3.80 (0.97)	21.33 (0.63)	7.09 (0.26)	5.02 (0.18)	10.76 (0.99)	289.01 (0.76)	92.09 (0.84)	14.56 (0.58)
2 × 35S:SPS2	4.57 (0.77)	21.48 (1.06)	8.16 (0.82)	5.43 (0.6)	12.76 (0.04)	264.20 (10.61)	79.58 (5.18)	14.40 (2.19)
2 × 35S:SPS3	3.43 (0.23)	19.95 (0.22)	7.03 (0.27)	5.61 (0.27)	9.57 (0.37)	282.29 (0.54)	88.76 (1.72)	14.84 (0.36)
2 × 35S:SPS4	5.09 (0.95)	21.89 (0.96)	8.24 (0.98)	5.74 (0.03)	11.42 (2.73)	288.84 (15.31)	95.26 (5.01)	16.27 (0.10)

Data are means ± SD of five independent plants per line

corresponding control lines. The increased stem height of the transformed plants was due to increased internode elongation as determined by the distance between the nodes of PI = 5 and PI = 15, and the number of internodes did not vary between wild type and transgenic lines (data not shown).

The increased plant growth rates were also evident in the diameter of the stem internodes immediately above PI = 15 (Figures 3 and 4), as determined by caliper measurements at harvest. All lines showed significantly greater stem diameter, corresponding to the increased stem height. This was not the case with the stem internode diameter at PI = 5 where only one line, 2 × 35S:SPS1, had a significantly greater diameter. The lack of significance in stem diameters at PI = 5 in the transformed lines is not surprising as this section is an actively elongating portion of the plant and developmentally is not sink tissue where one would expect the accumulation of metabolites, such as sucrose, which would be anticipated to be influenced by the over-expression of SPS.

The concomitant altered stem height and diameter accumulation translated into significant increases in total stem biomass of the AtSPS transgenic tobacco plants (Figure 5). Increases, up to 100%, in fresh weight were observed in all four lines selected for in-depth analysis. This increased biomass can not be ascribed to solute retention, as the improved biomass accumulation was maintained even when the harvested tobacco tissue was oven dried: 2 × 35S:SPS1 and 2 × 35S:SPS3 proved to be statistically different at $p = 0.05$, while 2 × 35S:SPS2 and 2 × 35S:SPS4 were significant at $p = 0.1$.

An analysis of the fibre properties of the transgenic tobacco compared with the corresponding controls revealed that the transgenic plants had statistically significantly longer fibres than the controls (Figure 6). The altered fibre property trends in the transgenic lines correlated with the increased stem height, and concur with the quantifiable changes in internodal length (elongation).

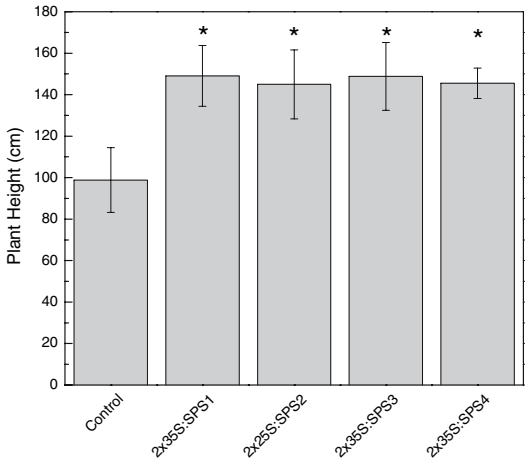

Fig. 2 Plant height at the onset of flowering from glasshouse-grown transgenic and nontransgenic control tobacco plants. Measurements are averages of ten individual plants for each independent transgenic line and control plants. Asterisk (*) denotes significant difference from control at $p < 0.05$, as determined by ANOVA analysis

Discussion

Differential expression of a family A arabidopsis SPS gene (SPS; EC 2.3.1.14) was achieved in both tobacco leaf and stems tissue by over-expression of the AtSPS gene under the regulation of the constitutive 2 × 35S promoter. Generally, higher transcript abundance was observed in leaves relative to the stem in all of the 2 × 35S:SPS transgenic lines evaluated. The elevated transcript abundance and associated elevated enzyme concentrations manifested significant enhancement in all growth parameters in all transformed lines investigated, namely height and diameter growth and biomass accumulation, as well as fibre length. However, the over-expression did not alter plant phenology and/or leaf morphology (size, shape, or number). The proposed role(s) for SPS in cell wall development and cellulose production is just one component of the regulation of carbon partitioning in the plant ascribed to SPS, and there are reports that demonstrate a relationship between SPS and plant growth. Haigler et al. (2000) observed altered fibre traits in cotton fibre cells over-expressing a spinach SPS gene. In tomato transformed with a maize SPS gene concurrent increased shoot and decreased root biomass were observed (Galtier et al., 1993), while antisense arabidopsis SPS showed a 50% reduction in plant growth (Strand et al., 2001). Additionally, SPS has been linked to a quantitative trait locus (QTL) controlling growth and yield in rice,

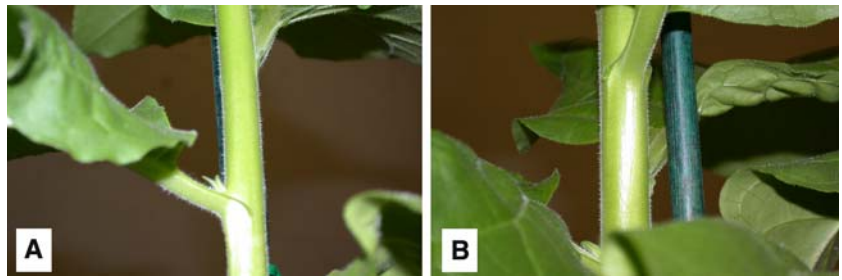

Fig. 3 Photos demonstrating differences in stem diameters at 4 weeks post planting, of (A) nontransgenic and (B) transgenic tobacco plants

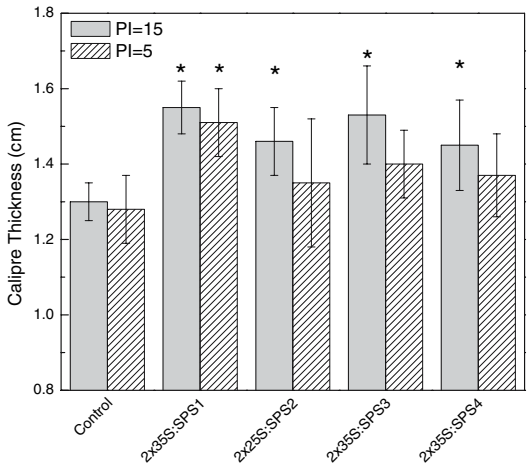

Fig. 4 Stem diameter ratios (diameters taken from stems sections PI = 5 and PI = 15) from glasshouse-grown transgenic and nontransgenic control tobacco. Measurements are averages of ten individual plants for each independent transgenic line and control plants. Asterisk (*) denotes significant difference from control at $p < 0.05$, as determined by ANOVA analysis

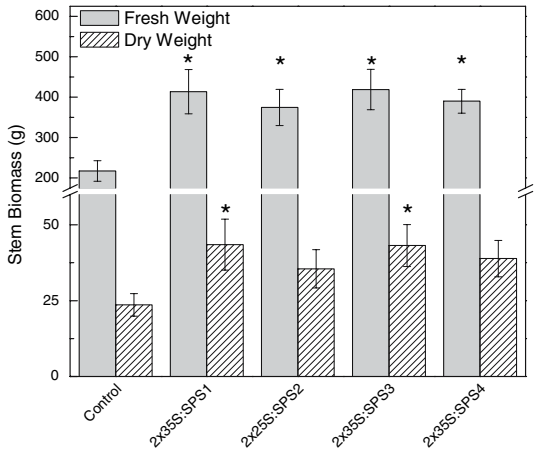

Fig. 5 Plant biomass from glasshouse-grown transgenic and nontransgenic control tobacco plants. Measurements are averages of ten individual plants for each independent transgenic line and control plants. Asterisk (*) denotes significant difference from control at $p < 0.05$, as determined by ANOVA analysis

and SPS-transgenic rice has been reported to grow taller than nontransformed plants (Castleden et al., 2004). Unfortunately, the exact role of SPS in plant growth and yield is not clearly understood as there are also reports of transgenic tomato plants expressing the maize SPS gene under the regulation of the rbcS promoter where no, or minor, changes in growth or biomass were observed (Ferrario-Méry et al., 1997), although other parameters such as flowering and fruit set were altered (Micallef et al., 1995). Furthermore, consistent changes among all transgenics is not always the case, as Laporte et al. (1997) reported increased biomass in only one of four SPS-transgenic tomato lines. Similar lack of growth enhancements were reported in independent studies involving tobacco (Baxter et al., 2003) and tomato (Worrell et al., 1991).

In the current study, tobacco plants transformed with an arabidopsis SPS gene demonstrated enhanced plant growth traits (stem height and diameter), elevated transcript abundance and SPS enzyme activity (2 of 4 lines), and substantial pooling of soluble stem sucrose content. The observed modifications were very comparable among lines evaluated, and therefore trends in phenotypic correlations were not obvious. However, it is clear that the overexpression of this Arabidopsis SPS gene in tobacco manifests quantifiable changes in transcript abundance and enzymatic activity in both leaf and stem tissue, and ultimately regulated plant growth and fibre development. Similar trends in transgenic plants harboring the exogenous SPS genes are not consistent in the literature, and the anomalies with the overexpression of SPS have, for example, previously been

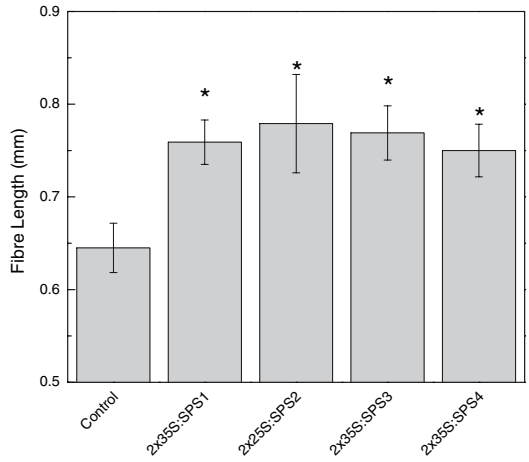

Fig. 6 Length weighted fibre length (PI = 15) from glasshouse-grown transgenic and nontransgenic control tobacco. Measurements are averages of ten individual plants for each independent transgenic line and control plants. Asterisk (*) denotes significant difference from control at $p < 0.05$, as determined by ANOVA analysis

ascribed to light activation of enzymatic function in transgenic tobacco expressing the maize SPS gene (Huber and Huber 1996). However, when the same gene was over-expressed in tomato, relatively little light induced activity was observed (Galtier et al., 1993). Additionally, in arabidopis, where endogenous SPS activity has not been shown to be light modulated, transformation with the maize SPS gene did confer light modulation of SPS activity (Signora et al., 1998).

Laporte et al. (2001) previously suggested that SPS-modulated enhanced growth is most likely due to altered partitioning of carbon, primarily between starch, sucrose and ionic compounds. More recently, Chen et al. (2005) demonstrated functional differences between SPS gene families in tobacco, where family A expression patterns were observed in all tissues, while family B and C were prevalent in reproductive organs and mature source leaves, respectively. Furthermore, it was shown by RNA interference that down-regulation of family A had no effect on leaf carbohydrate metabolism, while similar reductions of family C lead to increased leaf starch content (via impairment of starch mobilization). The findings of Chen et al. (2005) concur with our current findings that demonstrate that although elevated SPS activity was achieved in leaf tissue, the available soluble sucrose pools were not significantly altered. In contrast, the sucrose content of the tobacco stems were significantly elevated, suggesting that basal levels of leaf sucrose are maintained and all ''excess'' sucrose is translocated rapidly and consistently from source to sink tissue. Such a conclusion is further supported by fact that starch content is also very similar among the control and transgenic lines, and therefore there is no evidence of altered carbon partitioning to starch storage (at least during this period of the diurnal cycle—mid day)

In cells undergoing rapid cell wall thickening, Haigler et al. (2001) proposed that the main role of SPS is controlling fructose recycling to provide additional and continuous concentrations of available sucrose to SuSy and to remove fructose from the cellular pool as in an effective inhibitor of SuSy. The current finding again lend support to these claims which illustrate that the substantial intracellular pools of sucrose in stem tissue in some way manipulates fibre development and deposition, resulting in longer fibres lengths and consequently taller plants of greater biomass. The over-expression of exogenous SuSy has recently been shown to also positively impact stem height of transgenic tobacco (Coleman et al., 2006), and therefore supports such a hypothesis. Carbon partitioning, as suggested by Laporte et al. (2001), is not apparent in the current study, as the polymeric carbohydrates in the sink tissue (stem) are both slightly reduced (cellulose and starch) in comparison to the corresponding control tissues.

Previously, higher leaf sucrose levels were correlated with increased SPS activity in tomato, sugarcane, and arabidopsis (Worrell et al., 1991; Zhu et al., 1999; Signora et al., 1998, Murchie et al., 1999). In the current investigation, soluble leaf sucrose concentrations do not appear to be altered compared to the wild-type plants. However, the stem (sink tissue) sucrose concentrations were significantly elevated consistently in all lines, to greater than 118% in one line. While we have no definitive explanation for the differences between our results and those of the previous studies, it is possible that differences in either gene specificity, maize versus arabidopsis, promoter choice, or some specific regulatory response of the host plant (tobacco) contributed to these differences (i.e. protein phosphorylation or the putative tetrameric nature of the protein). Our data do clearly demonstrate a difference in stem carbohydrate

levels, an attribute which has not been investigated in any of the previous studies.

Much of the difficulty in understanding the effect of SPS through the over-expression of a SPS transgene is the very specific and complicated regulatory system of this enzyme and the extent this plays in transgene expression. For example, it has been proposed that SPS forms a complex with sucrose-phosphatase (Lunn and MacRae 2003) and that this complex may be critical in the synthesis of sucrose. SPS is also typically a low abundance protein (<0.1% of total protein) and relatively unstable (Huber and Huber, 1996). A final regulatory network involves the fact that SPS is encoded by a multigene family and is highly regulated by phosphorylation with two different kinases acting on different members of the SPS gene family (Lunn and MacRae, 2003). Furthermore, Laporte et al. (2001) suggested that the optimal activity for enhanced growth in transgenics is 2× the wild-type SPS activity level. Clearly, any component in this complex regulatory network could limit the recovery of a plant(s) with a clear phenotype.

The disparity in regulatory mechanism for the different members of the SPS gene family have led some investigators to speculate that the expression of maize B-family genes in dicots where A family genes predominate, may be the most successful means of manifesting altered phenotype and controlling carbon partitioning (Lunn and MacRae, 2003). However, to date, the literature has proven the maize gene to be highly variable in different backgrounds. Clearly, studies such as the present one are needed to determine if a monocot SPS gene is indeed any more or less variable in expression than a dicot SPS gene.

The modulation of complex variables such as plant growth, chemical composition and gene expression are of central interest to the plant biotechnology community. The current findings clearly demonstrate that the over-expression of SPS in plants has the potential to dramatically improve plant growth rates. It is less clear what potential the over-expression of SPS has to alter the production and partitioning of cell wall precursors such as sucrose and starch in sink tissue. However, there is no doubt that improving energy capture, conversion of radiant energy, and the allocation of carbon within the plant are promising areas for future plant improvement strategies. Equally important is the capacity to regulate cell wall ultrastructural properties (i.e. fibre traits), which present several key opportunities for biotechnological applications manipulating plant growth and development in several areas, including agriculture and forestry.

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References

Babb VM, Haigler CH (2000) Exploration of a role for sucrose phosphate synthase in cellulose synthesis during secondary cell wall deposition. In: Proceedings of Plant Biology 15–19 July, San Diego, CA. American Society of Plant Physiologists, Rockville, MD. Abstract 319. http://www.aspp.org/annual-meeting/pb-2000/2000.html

Baxter CJ, Foyer CH, Turner J, Rolfe SA, Quick WP (2003) Elevated sucrose phosphate synthase activity in transgenic tobacco sustains photosynthesis in older leaves and alters development. J Exp Botany 54:1813–1820

Braford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Castleden CK, Aoki N, Gillespie VJ, MacRae EA, Quick WP, Buchner P, Foyer CH, Furbank RT, Lunn JE (2004) Evolution and function of the sucrose-phosphate synthase gene families in wheat and other grasses. Plant Physiol 135:1753–1764

Chen S, Hajirezaei M, Börnke F (2005) Differential expression of sucrose-phosphate synthase isoenzymes in tobacco reflects their functional specialization during dark-governed starch mobilization in source leaves. Plant Physiol 139:1163–1174

Coleman HD, Ellis DD, Gilbert M, Mansfield SD (2006) Up-regulation of sucrose and UDP-glucose pyrophosphroylase impacts plant growth and metabolism. Plant Biotechnol J 4:87–101

Dalta RSS, Bekkaoui F, Hammerlindl JK, Pilate G, Dunstan DI, Crosby WL (1993) Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci 94:139–149

Delmer DP (1999) Cellulose biosynthesis: Exciting times for a difficult field of study. Annu Rev Plant Physiol Plant Molec Biol 50:245–276

Ferrario-Méry S, Murchie E, Galtier N, Quick WP, Foyer CH (1997) Manipulation of the pathways of sucrose biosynthesis on nitrogen assimilation in transformed plants to improve photosynthesis and productivity. In: Foyer CH, Quick WP (eds) A molecular approach to primary metabolism in higher plants. Taylor and Francis, London, pp 125–153

Fung RWM, Langenkämper G, Gardner RC, MacRae EA (2003) Differential expression within an SPS gene family. Plant Sci 164:459–470

Galtier N, Foyer CH, Huber J, Voelker TA, Huber SC (1993) Effects of elevated sucrose phosphate synthase activity on photosynthesis assimilate partitioning, and growth in tomato (*Lycopersicon esculentum* var UC83B). Plant Physiol 101:535–543

Geigenberger P, Reimholz R, Deiting U, Sonnewald U, Stitt M (1999) Decreased expression of sucrose phosphate synthase strongly inhibits the water stress-induced synthesis of sucrose in growing potato tubers. The Plant J 19:119–129

Geigenberger P, Reimholz R, Geiger M, Merio L, Canale V, Stitt M (1997) Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. Planta 201:502–518

Haigler CH, Holaday AS, Wu C, Wyatt BG, Jividen GJ, Gannaway JG, Cai WX, Hequet EF, Jaradat TT, Krieg DR, Martin LK, Strauss RE, Nagarur S and Tummala J (2000) Transgenic cotton over-expressing sucrose phosphate synthase produces higher quality fibres with increased cellulose content and has enhanced seedcotton yield. In: *Proceedings of Plant Biology, 15–19 July, San Diego, CA*. American Society of Plant Physiologists, Rockville, MD. Abstract 477. http://www.aspp.org/annual-meeting/pb-2000/2000.htm

Haigler CH, Ivanova-Datcheva M, Hogan PS, Salnikov VV, Hwang S, Martin K, Delmer DP (2001) Carbon partitioning to cellulose synthesis. Plant Molec Biol 47:29–51

Hood EE, Gelvin SB, Melcher LS, Hoekema A (1993) New agrobacterium helper plasmids for gene-transfer to plants. Transg Res 2:208–218

Horsch RB, Fry JE, Hoffmann NL, Eicoltz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Huber SC, Huber JL (1996) Role and regulation of sucrose phosphate synthase in higher plants. Annu Rev Plant Physiol Plant Molec Biol 47:431–444

Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD (2003) Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J Agric Food Chem 51:6178–6183

Im KH (2004) Expression of sucrose-phosphate synthase (SPS) in non-photosynthetic tissues of maize. Molec Cells 3:404–409

Iraqi D, Tremblay FM (2001) Analysis of carbohydrate metabolism enzyme and cellular contents of sugars and proteins during spruce somatic embryogenesis suggests a regulatory role of exogenous sucrose in embryo development. J Exp Botany 52:2301–2311

Kutschera U, Heiderich A (2002) Sucrose metabolism and cellulose biosynthesis in sunflower hypocotyls. Physiol Plantarum 114:372–379

Langenkämper G, Fung RWM, Newcomb RD, Atkinson RG, Gardner RC, MacRae EA (2002) Sucrose phosphate synthase genes in plants belong to three different families. J Molec Evol 54:322–332

Laporte MM, Galagan JA, Prasch AL, Vanderveer PJ, Hanson DT, Shewmaker CK, Sharkey TD (2001) Promoter strength and tissue specificity effects on growth of tomato plants transformed with maize sucrose phosphate synthase. Planta 212:817–822

Laporte MM, Galagan JA, Shapiro JA, Boersig MR, Shewmaker CK, Shakey TD (1997) Sucrose phosphate synthase activity and yield analysis of tomato plants transformed with maize sucrose phosphate synthase. Planta 203:253–259

Lunn JE, MacRae EA (2003) New complexities in the synthesis of sucrose. Curr Opin Plant Biol 6:208–214

Micallef BJ, Haskin KA, Vanderveer PJ, Roth K-S, Shewmaker CK, Sharkey TD (1995) Altered photosynthesis, flowering and fruiting in transgenic tomato plants that have an increased capacity for sucrose synthesis. Planta 196:327–334

Murchie EH, Sarrobert C, Contard P, Betsche T, Foyer CH, Galtier N (1999) Overexpression of sucrose-phosphate synthase in tomato plants grown with CO_2 enrichment leads to decreased foliar carbohydrate accumulation relative to untransformed controls. Plant Physiol Biochem 3:251–260

Murashige T, Skoog F (1962) A revised medium for rapid growth and biomass with tobacco tissue cultures. Physiol Plantarum 15:473–479

Salnikov VV, Grimson MJ, Delmer DP, Haigler CH (2001) Sucrose synthase localizes to cellulose synthesis sites in tracheary elements. Phytochemistry 57:823–833

Signora L, Galtier N, Skot L, Lucas H, Foyer CH (1998) Overexpression of sucrose-phosphate synthase in arabidopsis thaliana results in increased foliar sucrose/starch ratios and favours decreased foliar carbohydrate accumulation in plants after prolonged growth with CO_2 enrichment. J Exp Botany 49:669–680

Stitt M, Wilke I, Feil R, Heldt HW (1998) Coarse control of sucrose phosphate synthase in leaves: alterations of the kinetic properties in response to the rate of photosynthesis and the accumulation of sucrose. Planta 174:217–230

Strand Å, Zrenner R, Trevanion S, Stitt M, Gustafsson P (2001) Decreased expression of two key enzymes in the sucrose biosynthesis pathway, cytosolic fructose-1,6-bisphophatase and sucrose phosphate synthase, had remarkably different consequences for photosynthetic carbon metabolism in transgenic *Arabidopsis thaliana*. The Plant J 23:759–770

Sturm A (1999) Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. Plant Physiol 121:1–7

Worrell AC, Bruneau JM, Summerfelt K, Boersig M, Voelker TA (1991) Expression of a maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. The Plant Cell 3:1121–1130

van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A, Stiekema WJ (1995) pBINPLUS: An improved plant transformation vector based on pBIN19. Transg Res 4:288–290

Zhu YJ, Komor E, Moore PH (1997) Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose synthase. Plant Physiol 115:609–616